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Regulation of nicotinic receptor trafficking by the transmembrane Golgi protein UNC-50

Stefan Eimer[1,2,7,8], Alexander Gottschalk[3,7,9], Michael Hengartner[4,5,10], H Robert Horvitz[4,5], Janet Richmond[6], William R Schafer[3,11] and Jean-Louis Bessereau[1,2,*]

[1]Ecole Normale Supérieure, Biology Department, Paris, France, [2]INSERM, U789, Biologie cellulaire de la synapse, Paris, France, [3]Division of Biological Sciences, University of California, San Diego, La Jolla, CA, USA, [4]Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA, USA, [5]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA and [6]Department of Biology, University of Illinois, Chicago, IL, USA

Nicotinic acetylcholine receptors (AChRs) are pentameric ligand-gated ion channels that mediate fast synaptic transmission at the neuromuscular junction (NMJ). After assembly in the endoplasmic reticulum (ER), AChRs must be transported to the plasma membrane through the secretory apparatus. Little is known about specific molecules that mediate this transport. Here we identify a gene that is required for subtype-specific trafficking of assembled nicotinic AChRs in *Caenorhabditis elegans*. *unc-50* encodes an evolutionarily conserved integral membrane protein that localizes to the Golgi apparatus. In the absence of UNC-50, a subset of AChRs present in body-wall muscle are sorted to the lysosomal system and degraded. However, the trafficking of a second AChR type and of GABA ionotropic receptors expressed in the same muscle cells is not affected in *unc-50* mutants. These results suggest that, in addition to ER quality control, assembled AChRs are sorted within the Golgi system by a mechanism that controls the amount of cell-surface AChRs in a subtype-specific way.

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*Corresponding author. Ecole Normale Supérieure, INSERM, U789, Biologie cellulaire de la synapse, 46 Rue d'Ulm, Paris 75005, France. Tel.: +33 1 44 32 23 05; Fax: +33 1 44 32 36 54;
E-mail: jlbesse@biologie.ens.fr
[7]These authors contributed equally to this work
[8]Present address: European Neuroscience Institute (ENI), Center for Molecular Physiology of the Brain (CMPB), Goettingen 37077, Germany
[9]Present address: Institute for Biochemistry, Goethe-University, Frankfurt 60438, Germany
[10]Present address: Institute of Molecular Biology, University of Zurich, Zurich 8057, Switzerland
[11]Present address: MRC Laboratory of Molecular Biology, Cambridge CB2 0QH, UK

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Introduction

Nicotinic acetylcholine receptors (AChRs) belong to a family of structurally related ligand-gated ion channels that includes γ-aminobutyric acid (GABA), serotonin (5-HT), and glycine receptors (Corringer *et al*, 2000). AChRs mediate fast synaptic transmission at the neuromuscular junction (NMJ) and modulate neurotransmitter release in the brain (Gotti and Clementi, 2004). Recently, AChRs have also been shown to be expressed in non-neuronal cells where they control important cell functions such as proliferation, adhesion, migration, secretion, survival, and apoptosis (Wessler *et al*, 2003; Gotti and Clementi, 2004). These receptors are built by the assembly of individual subunits into pentamers, which can be either homo- or heteromeric in nature (Unwin, 2005). To be functional, AChRs must be assembled into pentamers and trafficked to the postsynaptic plasma membrane. Malfunction or misexpression of AChRs is involved in neuro-pathological processes such as schizophrenia, epilepsy, and tobacco addiction (Ryan, 1999; Gotti and Clementi, 2004; Singh *et al*, 2004), emphasizing the importance of control checkpoints to ensure appropriate cell-surface display of properly folded and assembled receptors.

AChR subunits assemble and oligomerize within the endoplasmic reticulum (ER) in a sequential and ordered manner (Smith *et al*, 1987; Green and Millar, 1995). However, in mouse muscle cells, only about 30% of the synthesized subunits reach the cell surface, suggesting that the assembly process is tightly controlled (Merlie and Lindstrom, 1983). Newly synthesized AChR subunits are immediately bound by the ER chaperones calnexin and Bip, from which they detach upon further maturation and assembly (Forsayeth *et al*, 1992; Gelman *et al*, 1995). Correct pentameric assembly is monitored by an ER retention signal in the first transmembrane (TM) segment present in each of the subunits, which is masked upon assembly of the pentamer (Wang *et al*, 2002), and by recognition of ER export motifs present in the large TM3 to TM4 intracellular loop (Keller *et al*, 2001; Ren *et al*, 2005). AChR subunits that do not fold or assemble properly are retained within the ER, and are rapidly degraded by the ER-associated degradation (ERAD) machinery (Wanamaker *et al*, 2003; Christianson and Green, 2004). Although the maturation and assembly processes in the ER seem to be inefficient, about 80% of the cellular nAChR are found at the cell surface at steady state (Devreotes and Fambrough, 1975; Gu *et al*, 1989). Once AChRs have reached the cell surface, they are clustered and metabolically stabilized at the synapse by attachment to the postsynaptic scaffold (Wang *et al*, 1999; Sanes and Lichtman, 2001) or by extracellular interactions (Gally *et al*, 2004). Despite our knowledge of the early events during AChR assembly and ER retention, molecules required for AChR cell-surface transport have yet to be identified. This is intriguing since several reports indicate that cell-type-specific

factors should exist that are necessary for AChR cell-surface expression (Sweileh *et al*, 2000)

Genetic screens performed in the nematode *Caenorhabditis elegans* have contributed to the identification of functional AChRs and molecules required for AChR assembly (Jones *et al*, 2005). ACh is the prominent excitatory neurotransmitter in *C. elegans* and is involved in most physiological functions such as locomotion, feeding, and mating. Genome sequencing revealed up to 42 genes potentially encoding AChR subunits (Jones and Sattelle, 2004). An AChR present at the *C. elegans* NMJ was first identified and characterized on the basis of its sensitivity to the nematode-specific nicotinic agonist levamisole (Lewis *et al*, 1980). This drug causes muscle hypercontraction and death at high concentration. A screen for resistance to levamisole identified mutations in the genes *unc-29*, *unc-38* and *unc-63* that encode obligatory subunits of the levamisole-sensitive AChR (Lev-AChR) expressed in body-wall muscles (Fleming *et al*, 1997; Culetto *et al*, 2004). In addition, the loss of LEV-1 or LEV-8 AChR subunits causes a partial resistance to levamisole, suggesting that these subunits are present in a subset of the levamisole receptors or are functionally redundant (Fleming *et al*, 1997; Towers *et al*, 2005). Mutants in Lev-AChRs exhibit a slow and uncoordinated movement, but are still able to move, since body-wall muscles express a second type of nicotinic AChR (Richmond and Jorgensen, 1999). *acr-16* encodes an essential subunit of this receptor, which is likely to represent an α7-like homopentameric receptor (Ballivet *et al*, 1996; Francis *et al*, 2005; Touroutine *et al*, 2005).

Genetic strategies also identified proteins required for AChR synthesis or cell-surface expression. The gene *ric-3* was isolated in a screen for suppressors of the neuronal degeneration caused by a gain-of-function mutation in the *C. elegans* AChR composed of the DEG-3 and DES-2 subunits (Halevi *et al*, 2002). *ric-3* encodes an integral membrane protein localized in the ER, and is required for the maturation of all AChRs analyzed in *C. elegans* so far. Subsequently, mammalian homologs of RIC-3 have also been identified and shown to be involved in the functional maturation of different types of AChRs (Halevi *et al*, 2002, 2003). Another locus, *unc-50*, was identified in a screen for resistance to levamisole and was mapped to a genetic interval that did not contain genes potentially encoding AChR subunits (Lewis *et al*, 1980). However, *unc-50* mutants displayed the same levamisole resistance and uncoordinated phenotype as mutations in the Lev-AChR subunits (Lewis *et al*, 1980). Furthermore, membrane extracts of *unc-50* mutants were demonstrated to lack binding sites for labeled amino-levamisole in a ligand binding assay (Lewis *et al*, 1987). These data suggested that UNC-50 might be required for the synthesis of functional Lev-AChRs.

Here we demonstrate that UNC-50 belongs to an evolutionarily conserved protein family of integral membrane proteins (Fitzgerald *et al*, 2000; Chantalat *et al*, 2003). In *C. elegans*, UNC-50 is ubiquitously expressed and localized to the Golgi system. We show that UNC-50 is required in body-wall muscles for the trafficking of the assembled Lev-AChR to the NMJ. In *unc-50* mutants, the Lev-AChR is rapidly degraded by the lysosomal system after receptor assembly. This late degradative pathway represents a novel regulatory step to control the biosynthesis of a specific subset of AChRs.

Results

unc-50 mutants lack Lev-AChRs at the cell surface

unc-50 mutants were initially isolated on the basis of impaired locomotion (Brenner, 1974), and were subsequently shown to be strongly resistant to the nicotinic agonist levamisole (Lewis *et al*, 1980). These two phenotypes suggest that *unc-50* mutants lack functional Lev-AChR at NMJs. To test this prediction we recorded the electrophysiological response of body-wall muscles to pressure-ejected levamisole in the wild type and *unc-50* mutants. In contrast to the wild type, *unc-50* mutants (alleles *x515* and *x47*) exhibited no response to levamisole (Figure 1A). The lack of response to levamisole was identical to that seen in mutants lacking the AChR subunits UNC-29, UNC-38 or UNC-63 (Richmond and Jorgensen, 1999; Culetto *et al*, 2004). Besides the Lev-AChR, a second AChR encoded by *acr-16* is present at *C. elegans* NMJs. This ACR-16-containing receptor is insensitive to levamisole but sensitive to nicotine. To assess the effect of *unc-50* mutations on this receptor, we recorded the response

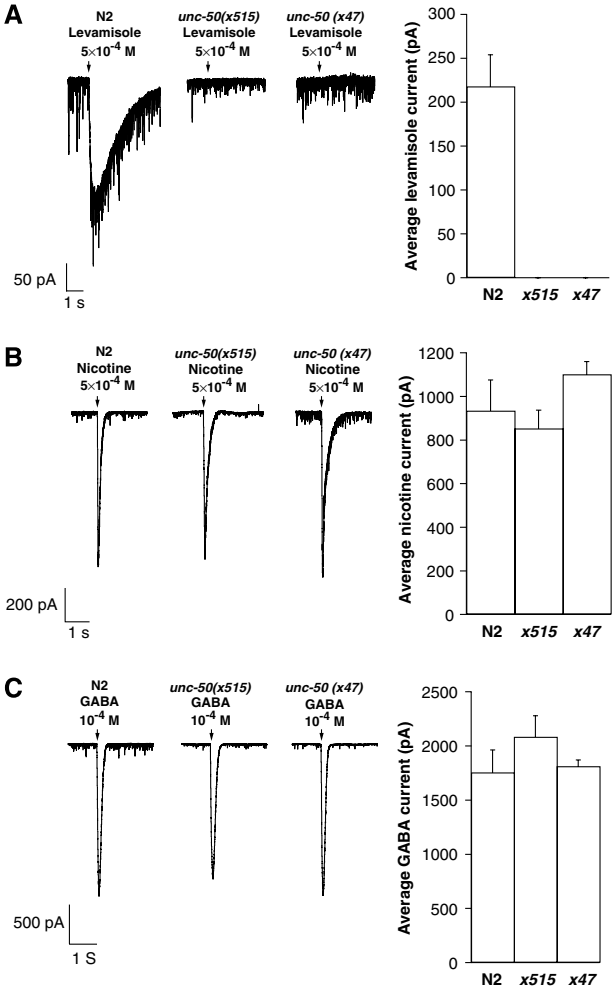

Figure 1 Body-wall muscles of *unc-50* mutants do not respond to levamisole, however the response to nicotine and GABA are unaffected. The electrophysiological responses of wild type (N2) and *unc-50* mutant (alleles *x515* and *x47*) body-wall muscles to pressure ejection of 500 μM levamisole (**A**), 500 μM nicotine (**B**), and 100 μM GABA (**C**) are shown. The arrow marks drug application. Results are presented as the means of independent experiments. Error bars represent the standard error of the mean (s.e.m.).

of body-wall muscles to nicotine and found no difference between wild-type and *unc-50* mutant animals (Figure 1B). Analysis of ACR-16-dependent evoked response in muscle cells following nerve stimulation was similar in wild-type and *unc-50* mutant animals (Supplementary Figure 1), hence demonstrating that UNC-50 is dispensable for expression and synaptic targeting of ACR-16-containing receptors. *C. elegans* muscles are also innervated by GABAergic motoneurons. At GABAergic NMJs, GABA activates an anionic $GABA_A$ receptor encoded by the *unc-49* gene. Electrophysiological responses to GABA in wild type and in *unc-50* mutants were similar (Figure 1C). Together, these results demonstrate that loss of UNC-50 function selectively eliminates the expression of functional Lev-AChRs, but does not affect the expression of other ligand-gated ion channels at the NMJ.

UNC-50 could either be required for cell-surface expression of the Lev-AChRs or for the function of these receptors at the cell surface. To distinguish between these two possibilities, we used a technique that allows the specific detection of cell-surface receptors in living *C. elegans* animals (Gottschalk *et al*, 2005) (see Supplementary data). Injection of fluorescently labeled antibody into wild-type animals expressing either levamisole or $GABA_A$ epitope-tagged receptor subunits generated a punctate staining pattern (Figure 2A and C, respectively), similar to the staining pattern of endogenous receptors at the NMJs (Gally and Bessereau, 2003; Gally *et al*, 2004). However, no signal was detected in *unc-50* mutants expressing a tagged LEV-1 subunit, suggesting that no Lev-AChRs were present at the cell surface. In animals heterozygous for the *unc-50* mutation, the signal of the tagged LEV-1 subunit was reduced by about 30% (Figure 2A and B). Consistently, electrophysiological recording of *unc-50/+* animals showed a levamisole response reduced by approximately 50% (data not shown). This result suggests that UNC-50 might be a limiting factor for cell-surface expression of the Lev-AChR. In contrast, the $GABA_A$ receptor was expressed at wild-type levels at inhibitory NMJs in *unc-50* mutants (Figure 2C and D). Therefore, the lack of UNC-50 specifically prevents the cell-surface expression of the Lev-AChR.

Lev-AChRs are not detectable in unc-50 mutants

To determine the fate of Lev-AChRs in *unc-50* mutants, we used an antibody directed against the AChR subunit UNC-29. In wild-type animals, clustered receptors were detected at NMJs along the ventral and dorsal nerve cords, and in the nerve ring (Figure 3A; Gally *et al*, 2004). In contrast, there was no staining detectable by immunofluorescence in *unc-50* mutants, either at synaptic sites or in intracellular compartments, despite cell permeabilization during the staining procedure (Figure 3A). However, since we knew that endogenous Lev-AChRs escape detection if they are not clustered (Gally *et al*, 2004), this result did not rule out the possibility that receptors were diffusely distributed in intracellular membranes. To test this possibility, we analyzed isolated membrane fractions by Western blot experiments. In contrast to the wild type, there were no Lev-AChRs detectable in the membrane fractions of the *unc-50* mutants (Figure 3B).

To determine whether the lack of Lev-AChR expression was due to a transcriptional or post-transcriptional requirement for UNC-50, we analyzed the mRNAs of the Lev-AChR subunits by semi-quantitative RT–PCR. We could not detect significant differences in the expression of the subunits between wild type and *unc-50* mutants (data not shown).

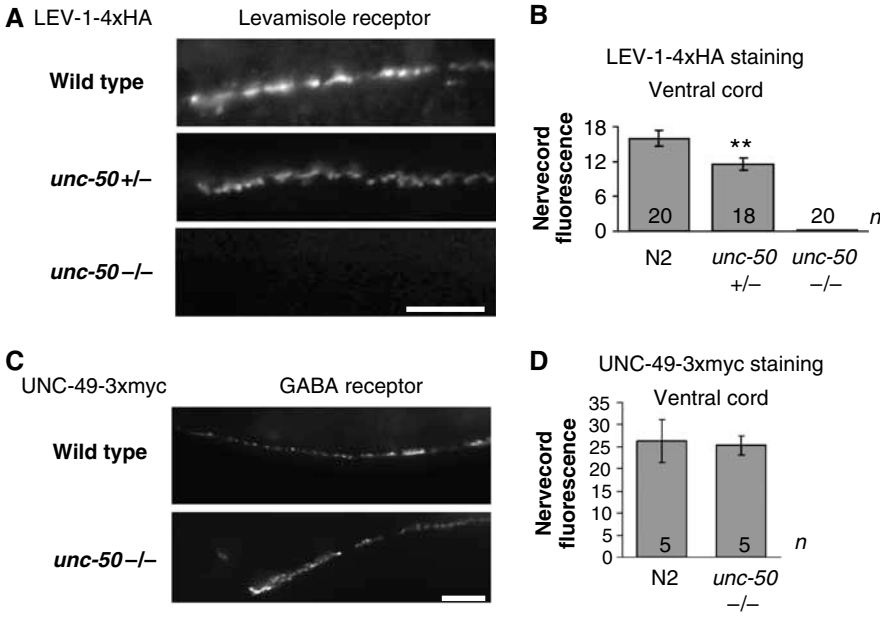

Figure 2 *unc-50* mutants do not display the Lev-AChR at the cell surface. Wild-type, *unc-50(e306)* heterozygote and *unc-50(e306)* mutant animals were engineered to express the C-terminally tagged LEV-1-4xHA Lev-AChR subunit (**A, B**). Similarly wild-type and *unc-50(e306)* mutant animals were engineered to express the UNC-49-3xmyc $GABA_A$ receptor subunit (**C, D**), and subsequently, fluorescently labeled antibodies against the respective epitopes were injected into the body cavity. After a recovery period of 6 h, in which the body cavity was cleared of unbound antibodies, the signal resulting from antibodies bound to the epitope-tagged receptors at the cell surface was measured and quantified. The signals originating from the ventral cord NMJs are shown in panels A and C, and quantified in panels B and D, respectively. The number of animals counted is indicated and the error bars represent the standard error of the mean (s.e.m.). $^{**}P<0.01$, *t*-test. The scale bars correspond to 10 μm.

Altogether, these data indicate that UNC-50 is required at a post-transcriptional step for expression of the levamisole-sensitive receptor in body-wall muscle cells.

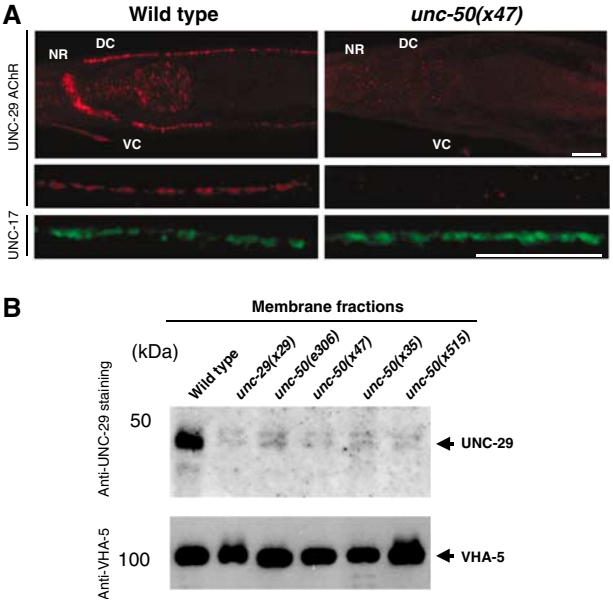

Figure 3 No Lev-AChR can be detected in *unc-50* mutants by immunostaining and Western blot analysis. (**A**) Wild-type (N2) and *unc-50(x47)* mutant young adult animals were freeze fractured, fixed in methanol/acetone and immunostained with polyclonal antibodies against the Lev-AChR subunit UNC-29. Antibodies to the ACh vesicular transporter UNC-17 were used to detect presynaptic terminals along the nerve cord. The positions of the nerve ring (NR), dorsal-, and ventral cord (DC and VC) NMJs are indicated. The scale bar represents 20 μm. (**B**) Total membrane fractions of staged L4 wild-type worms, *unc-29*, and *unc-50* mutants were size fractionated by SDS gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was sequentially probed with antibodies against UNC-29 and VHA-5. The TM subunit of the vacuolar ATPase VHA-5 was used to normalize the membrane fractions.

UNC-50 acts after receptor assembly

From the data obtained in the mammalian system, we know that the assembly of heteromeric AChRs is highly inefficient and active pathways exist at the ER level for their degradation (Green and Millar, 1995; Wanamaker *et al*, 2003; Christianson and Green, 2004). The lack of detectable Lev-AChR in *unc-50* mutants could thus be due to a translational block of Lev-AChR subunit-encoding mRNAs, or a failure in receptor assembly, resulting in rapid receptor degradation. In order to determine the fate of partially assembled Lev-AChRs in *C. elegans* muscles, we analyzed the receptor levels in mutants in which one of the subunits, UNC-38, UNC-63 or LEV-1, was missing. Interestingly, unassembled or partially assembled Lev-AChRs could be stably detected by Western blot analysis in these mutants at levels comparable to those in the wild type (Figure 4A). Since all Lev-AChR subunits contain in their first TM domain an ER retention signal conserved in vertebrate AChR subunits (Wang *et al*, 2002), it is predicted that these unassembled receptors are retained within the ER. In agreement with this, we were never able to detect unassembled Lev-AChRs at the cell surface either by immunostaining of fixed animals or by *in vivo* antibody injections to label tagged receptors at the cell surface (data not shown).

The fact that unassembled Lev-AChRs can be stably detected by Western blot experiments allowed us to test whether UNC-50 acts before or after receptor assembly. If UNC-50 is required before AChR assembly, impairing *unc-50* in animals missing one of the levamisole AChR subunits should cause disappearance of unassembled receptors. Conversely, if UNC-50 is required for the transport of the Lev-AChR after assembly, mutating *unc-50* should not affect the expression level of unassembled receptors. As shown in Figure 4B, the Lev-AChR subunit UNC-29 can be detected by Western blot analysis at similar levels in the membrane fractions of *unc-63* and *unc-63*; *unc-50* double mutants ($72 \pm 20\%$, $n = 6$). These results suggest that UNC-50 acts at

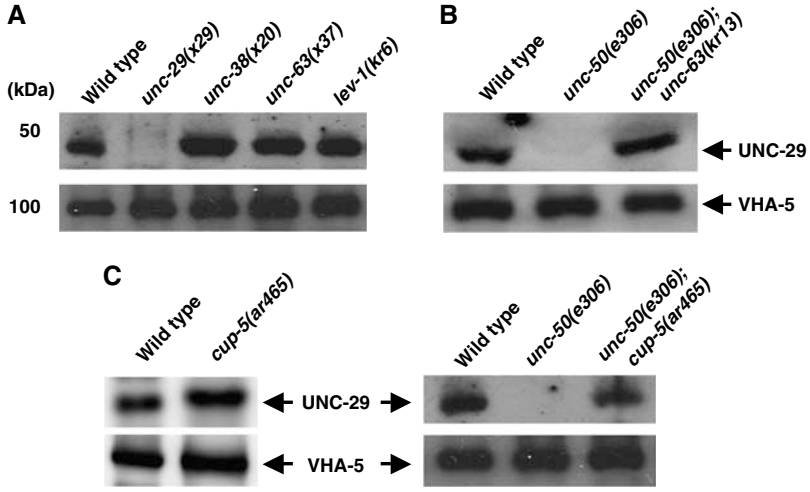

Figure 4 Total membrane fractions from staged L4 hermaphrodites of the indicated genotypes were size separated, blotted, and probed with antibodies against the Lev-AChR subunit UNC-29 and against the vacuolar ATPase subunit VHA-5 as a loading control. (**A**) The unassembled Lev-AChR is stable and can be detected in the mutants *unc-38(x20)*, *unc-63(x37)*, and *lev-1(kr6)* that lack one of the receptor subunits. While membrane extracts of *unc-29* mutants exhibit no detectable UNC-29 staining ($1.1 \pm 0.6\%$ ($n = 4$)), wild-type levels of staining are seen in *unc-38* ($115 \pm 18\%$ ($n = 3$)), *unc-63* ($101 \pm 11\%$ ($n = 2$)), and *lev-1* ($107 \pm 25\%$ ($n = 2$)) mutants. (**B**) UNC-50 acts after Lev-AChR assembly. The unassembled receptor can be detected in *unc-50*; *unc-63* double mutants and not in *unc-50* mutants. (**C**) The Lev-AChR is degraded by the lysosomal system. UNC-29 levels in *cup-5* mutants ($110 \pm 6\%$ ($n = 2$)) are comparable to wild-type N2 animals (100% ($n = 2$)), but mutations in *cup-5* stabilize the Lev-AChR in an *unc-50* mutant background.

a post-assembly step during trafficking of the Lev-AChR to the synapse.

Lev-AChRs are degraded by the lysosomal system in unc-50 mutants

Our results indicated that Lev-AChR subunits were expressed normally in *unc-50* mutants, were able to assemble and potentially leave the ER, but were then mis-addressed and transported aberrantly within the cell. This inappropriate targeting within the cells would lead to the efficient degradation of the Lev-AChR in *unc-50* mutants. It has been shown that TM proteins that leave the ER can be degraded by the lysosomal system (Blondel *et al*, 2004; Pizzirusso and Chang, 2004; Kim *et al*, 2005). To determine whether assembled Lev-AChRs are degraded by lysosomes in *unc-50* mutants, we introduced a *cup-5* mutation into the *unc-50* mutant background. The *C. elegans* CUP-5 protein is required for the formation of lysosomes from endosomal–lysosomal hybrid organelles that are generated by late endosome to lysosome transport (Treusch *et al*, 2004). As a result, *cup-5* mutants exhibit giant endosomal–lysosomal hybrid organelles that have reduced lysosomal activities, although the delivery of substrates to these structures occurs normally. Since complete loss of function of CUP-5 leads to embryonic lethality (Hersh *et al*, 2002), we used the viable *cup-5(ar465)* point mutation that is expected to retain low levels of CUP-5 activity. As shown in Figure 4C, the levels of UNC-29 are comparable between wild-type and *cup-5(ar465)* mutant animals. However, introduction of *cup-5(ar465)* in *unc-50* mutants causes a reappearance of the Lev-AChR subunit UNC-29 (Figure 4C, $28 \pm 3\%$ of wild-type levels, $n = 3$ independent experiments), despite residual lysosomal function in *cup-5(ar465)*. Despite the reappearance of detectable UNC-29 in Western blot experiments, *unc-50; cup-5* double mutants remain as uncoordinated and levamisole resistant as *unc-50* single mutants (data not shown), indicating that Lev-AChRs are not rerouted to the cell surface and are still delivered to lysosomes in a *cup-5* mutant background. Together, these results suggest that in the absence of UNC-50 Lev-AChRs enter the lysosomal compartment and are efficiently degraded by the lysosomes.

unc-50 encodes an evolutionarily conserved integral membrane protein

To gain insight into the role of UNC-50 in the trafficking of nicotinic receptors, we cloned the *unc-50* gene (Supplementary Figure 2). *unc-50* is the downstream gene of an operon, and corresponds to the ORF T07A5.2 on chromosome III (Figure 5A). Sequencing of two cDNA clones of *unc-50* (provided by Y Kohara) revealed that *unc-50* encodes a protein of 301 amino acids (Figure 5B) that is conserved in its gene structure and sequence between *C. elegans* and the related nematodes *C. briggsae* and *C. remanei* (data not shown). Nearly all *unc-50* mutations found in T07A5.2 are nonsense mutations, deletions or insertions leading to frame shifts expected to severely impair UNC-50 function (Figure 5A and 5B; Supplementary Table 1).

The protein encoded by *unc-50* is conserved throughout evolution from yeast to humans (Figure 5B). Orthologs of UNC-50 can be found in simple eukaryotic organisms like *Entamoeba histolytica* HM-1, but not in bacteria (Supplementary Figure 3). No other UNC-50-like protein could be identified in *C. elegans*. Interestingly, we were also unable to detect a second UNC-50-like protein in organisms such as *Arabidopsis* and zebrafish, in which a genome duplication has taken place. This suggests that UNC-50-like proteins provide a conserved and unique function in each organism.

Hydropathy analysis of UNC-50 and its orthologs in other organisms showed that this protein family contains five TM domains (Supplementary Figure 4). The N-terminus is predicted to reside in the cytoplasm, whereas the short C-terminus is located in a luminal compartment (Figure 5C). This topology is experimentally supported for the *Saccharomyces cerevisiae* homolog GMH1 by proteinase K protection assays of membrane fractions (Chantalat *et al*, 2003). Therefore, the largest domains of UNC-50, as well as the amino acids that are conserved among all species, are facing the cytoplasm (Figure 5C). This makes it likely that a conserved interaction surface is located at the cytosolic side of UNC-50.

UNC-50 is required in body-wall muscles for Lev-AChR trafficking

Based on *unc-50*::*gfp* transcriptional and translational fusions, *unc-50* is expressed ubiquitously from early embryogenesis to adulthood (Supplementary Figure 5). To determine whether UNC-50 is required only in body-wall muscles for Lev-AChR expression, we expressed UNC-50 under the control of the muscle-specific *myo-3* promoter. *unc-50* mutants exhibit uncoordinated slow movement and complete resistance to levamisole, identical to mutants expressing no Lev-AChR subunits (Figure 6A and B). Expression of the *myo-3*::*unc-50* construct was sufficient to completely rescue levamisole sensitivity (Figure 6A) and locomotory defects (Figure 6B). Immunostaining experiments performed on rescued *unc-50* mutants indicated that Lev-AChRs were clustered at NMJs (data not shown). Therefore, UNC-50 is cell-autonomously required in body-wall muscles for transport of Lev-AChRs to the cell surface.

However, these experiments did not rule out the possibility that UNC-50 might be required during development to generate structures required for the AChR trafficking rather than being necessary for its transport *per se*. To assess the temporal requirement of *unc-50* function during muscle development, we placed the *unc-50* cDNA under the control of the heat-shock-inducible promoter *hsp-16.48*. Transgenic *unc-50* mutant animals raised to adulthood at 20°C without a heat shock exhibited the same uncoordinated movement and resistance to levamisole as *unc-50* mutants (Figure 6C). These adult animals were then heat-shocked and assayed after 8 h for movement and levamisole sensitivity. Acute expression of UNC-50 in fully differentiated body-wall muscle was sufficient to rescue the uncoordinated movement and to render the transgenic animals sensitive to levamisole (Figure 6C). This experiment indicates that UNC-50 is a necessary factor for the cell-surface trafficking of the Lev-AChR in muscle and is not required for developmental processes within the muscle.

UNC-50 is localized to the Golgi system

To gain further insight into how UNC-50 mediates the transport of the Lev-AChR, we determined its intracellular localization. UNC-50 was fused to different fluorescent protein tags

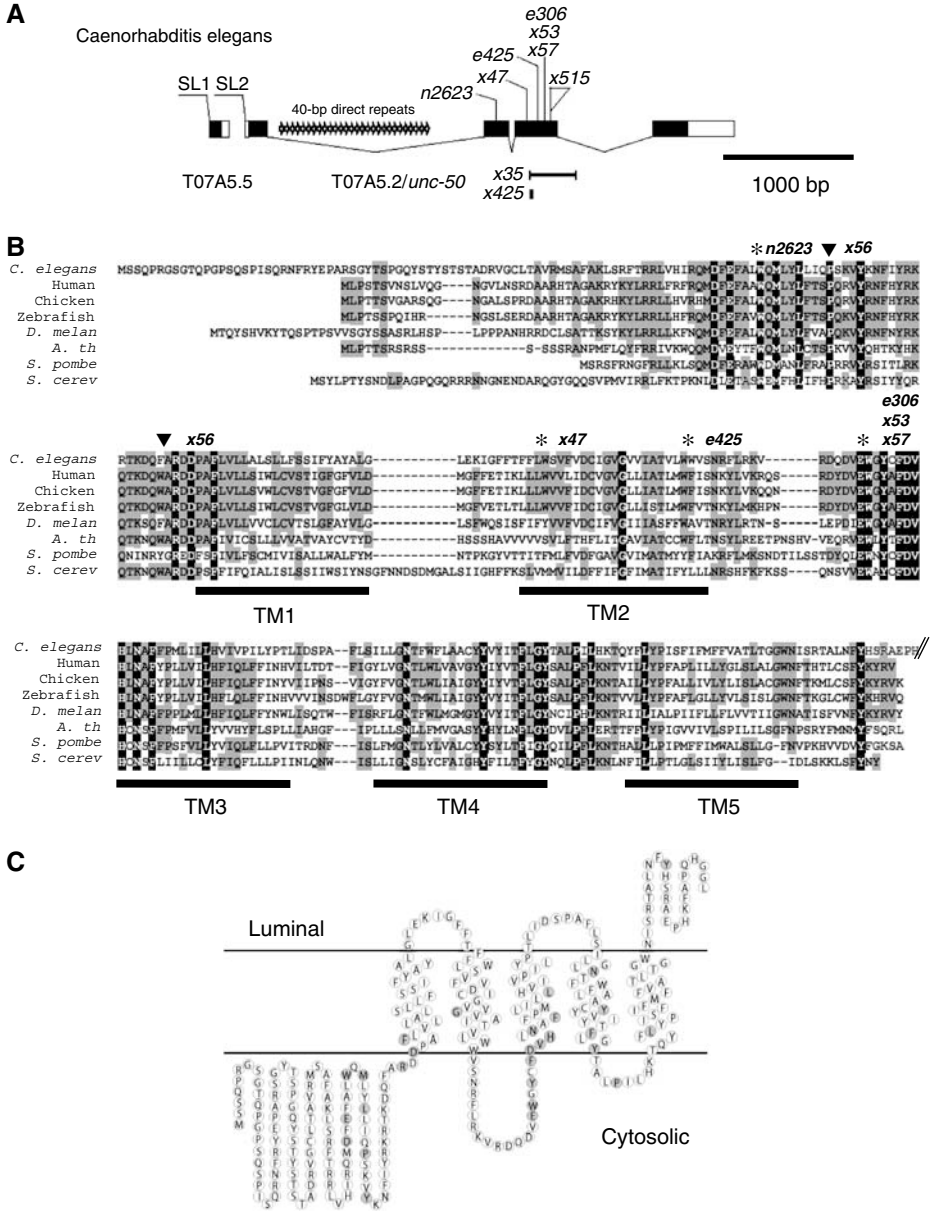

Figure 5 *unc-50* encodes an evolutionarily conserved integral membrane protein. (**A**) Genomic organization of T07A5.2/*unc-50*. *unc-50* is part of an operon, but all *unc-50* alleles carry mutations in the gene T07A5.2. The black regions represent coding regions, while white parts represent the untranslated regions. Splice leaders are indicated. (**B**) ClustalX alignment of UNC-50 with its orthologs from yeast to humans. The locations of the predicted TM regions are indicated. Residues conserved between all species are highlighted in black and conserved residues between most species in gray. The positions of the mutations detected in *unc-50* alleles are indicated above the protein sequence, asterisks correspond to stop mutations while the triangles mark the two point mutations found in the allele *x56*. The GenBank accession numbers for the sequences used can be found in the Supplementary Figure 3. The C-terminus of UNC-50 is truncated in this panel, but is shown in panel C. (**C**) Proposed membrane topology of UNC-50. The residues conserved between all species are underlined in gray.

and expressed in body-wall muscles. Fusions of fluorescent proteins to either the N- or C-terminus of UNC-50 were completely functional as they were able to rescue *unc-50*-associated phenotypes. In addition, N- and C-terminal UNC-50 fusion proteins showed the same vesicular staining pattern irrespective of the nature of the fluorescent protein tag (data not shown). As shown in Figure 7A, an mRFP-UNC-50 fusion protein expressed in body-wall muscles displayed a punctate distribution throughout the cytoplasm of the muscle cell. This staining is typical of the Golgi apparatus in *Drosophila* tissues or muscle myotubes (Gu *et al*, 1989; Rabouille *et al*, 1999). Indeed, a similar vesicular staining pattern is obtained with the medial Golgi marker α-Mannosidase II (Figure 7B). When confocal stacks were overlaid, mRFP-UNC-50 and Mannosidase II-GFP staining showed $91 \pm 3\%$ ($n = 4$) colocalization (Figure 7C). By contrast, no overlap was observed between mRFP-UNC-50 and the GFP-cytochrome *b5* (cb5) fusion, which is ER resident. This demonstrates that at steady state UNC-50 is mainly localized to the Golgi system in *C. elegans*. However, UNC-50 does not seem to be required for the maintenance of the ER or Golgi structures, since the distribution of the GFP-cb5 and Mannosidase II-GFP are

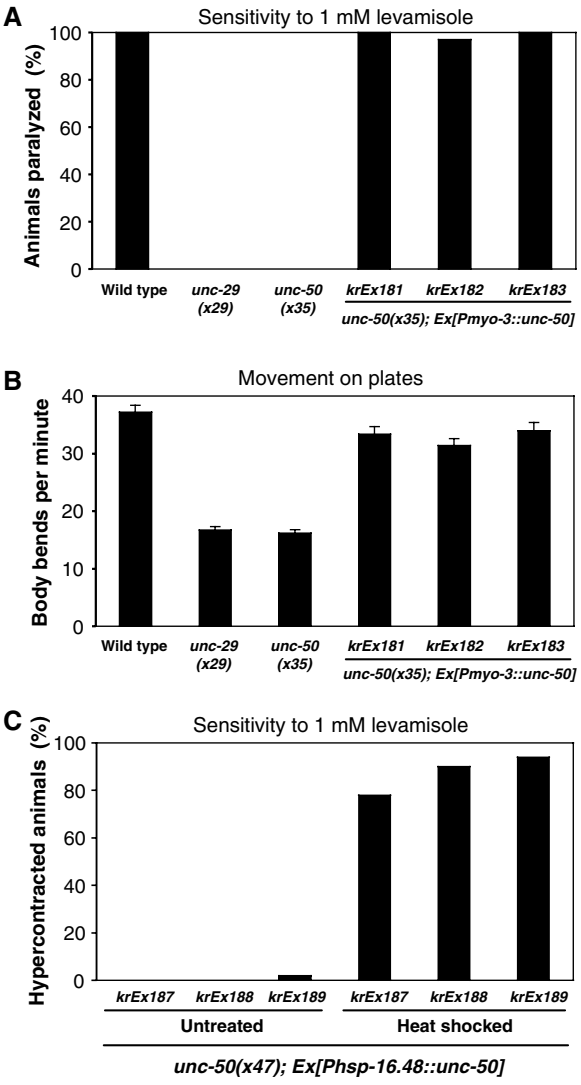

Figure 6 Muscle-specific expression of *unc-50* cDNA is sufficient to rescue the resistance to levamisole (**A**) and the uncoordinated movement phenotype (**B**) of *unc-50* mutant animals. Transgenic *unc-50* hermaphrodites express the *unc-50* cDNA under the control of the muscle-specific *myo-3* promoter from an extrachromosomal array. (**C**) Conditional expression of the *unc-50* cDNA under the control of the heat-shock promoter is able to rescue the levamisole resistance after induction by heat shock in adult animals carrying the transgene.

similar to wild type in *unc-50* mutants (Supplementary Figure 6). Golgi residence has also been shown for the yeast and human ortholog of UNC-50 (Chantalat *et al*, 2003), suggesting that UNC-50-like proteins localize to the Golgi system in general.

UNC-50 can interact with the Sec7 domain-containing Arf-GEF Gea1

GMH1 is the *S. cerevisiae* ortholog of UNC-50. Yeast two-hybrid and immunoprecipitation experiments showed that GMH1 binds to Gea1/2p, the large Sec7 domain-containing guanine exchange factors (GEFs) of the small GTPase Arf (Chantalat *et al*, 2003). Gea1/2p, and their mammalian ortholog GBF1, associate with early components of the secretory pathway (Chantalat *et al*, 2003; Niu *et al*, 2005; Zhao *et al*, 2006). GBF1-dependent Arf activation might regulate the recruitment of the COPI coat protein complex which is necessary for anterograde and retrograde transport processes between ER and Golgi and within the Golgi system (Shima *et al*, 1999; Garcia-Mata *et al*, 2003). Since UNC-50-like molecules, Arf GTPases and Sec7 domain-containing GEFs are conserved between yeast and *C. elegans* (Jackson and Casanova, 2000; Chantalat *et al*, 2003), we hypothesized that the interaction between UNC-50 and Gea1/2p Arf GEFs might be conserved, as has been shown for the yeast and human UNC-50 orthologs (Chantalat *et al*, 2003). To test this possibility we fused UNC-50 to the GAL4 transcriptional activation domain and assayed its interaction with the yeast Arf GEF Gea1 using the yeast two-hybrid system. As shown in Figure 8, UNC-50, like the yeast Gmh1 and the human hGmh1, specifically interacted with the C-terminus of the yeast Arf GEF Gea1p. However, the interactions between *C. elegans* and human UNC-50 molecules and the yeast Gea1p were weaker than that seen between the yeast Gmh1p and the yeast Gea1p (Figure 8). Interactions were lost when using Gea1L862S, a mutation that was shown to strongly reduce the interaction with Gmh1p (Chantalat *et al*, 2003) (data not shown). This conserved interaction of UNC-50 with a class of large Sec7 domain-containing Arf GEFs suggests that UNC-50 is also likely to be involved in Arf activation at Golgi membranes, and subsequently in COPI-dependent transport processes.

Discussion

Trafficking receptors to and from synapses is an efficient way to regulate synaptic strength. General factors of the membrane protein synthesis machinery play a well-documented role in the assembly and intracellular transport of ionotropic receptors, but only few factors are known to specifically regulate the biosynthesis of a defined receptor. Using a mutant identified in a screen for resistance to the paralyzing and lethal effects of the nicotinic agonist levamisole in *C. elegans*, we identified and characterized an integral membrane protein, UNC-50, that is required specifically for the cell-surface trafficking of AChRs sensitive to levamisole. We have shown that UNC-50 is localized to the Golgi system and interacts with Arf-GEFs. In the absence of UNC-50, the assembled Lev-AChR is sorted to a lysosomal degradative pathway. Despite the enormous complexity of AChR repertoire that can be expressed in a cell, regulation of the UNC-50-dependent pathway would provide a means to specifically regulate the biosynthesis of one defined receptor subtype.

UNC-50 might act as a membrane receptor for large Sec7 domain-containing Arf-GEFs

UNC-50 belongs to an evolutionarily conserved protein family that is found in all eukaryotic organisms. Initial transfection experiment using the rat UNC-50 ortholog named UNCL suggested that UNCL localized to the ER and the nuclear membrane in SaOS-2 osteosarcoma cells (Fitzgerald *et al*, 2000). However, in *C. elegans* we observed that tagged UNC-50 proteins able to rescue the *unc-50* mutant phenotypes localized to the Golgi at steady state. Results similar to ours have recently been reported for yeast and human UNC-50 orthologs. The yeast ortholog of *unc-50*, *GMH1*, was initially identified in a multicopy suppressor screen of growth defects caused by mutation of the genes

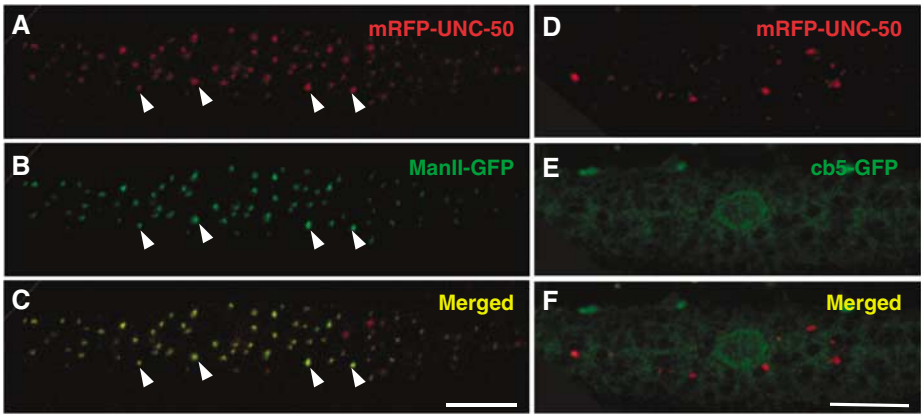

Figure 7 UNC-50 localizes to the Golgi system. (**A–C**) mRFP-UNC-50 fusion proteins display a vesicular staining throughout the cytoplasm of a body-wall muscle cell, as shown. This largely overlaps the staining of the Golgi-resident Mannosidase II-GFP fusion protein. Representative Golgi structures are marked by arrowheads. (**D–E**) The mRFP-UNC-50 fusion protein does not overlap with an ER-localized GFP-cb5 protein. (The scale bar = 10 μm.)

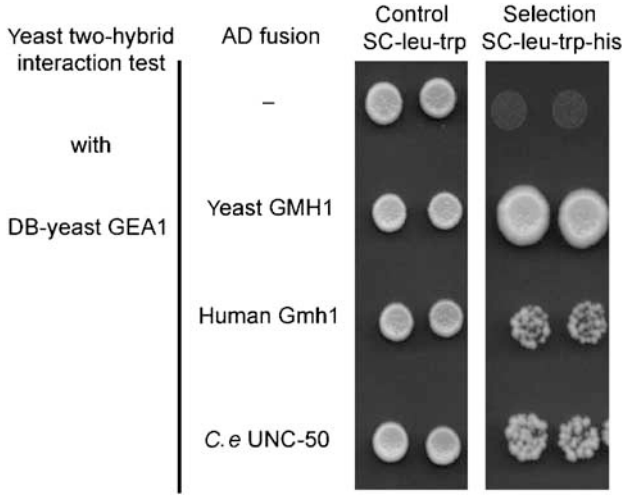

Figure 8 UNC-50 interacts with the SEC7 domain-containing Arf GEF Gea1p. Yeast two-hybrid interaction test of a yeast DB-GEA1 fusion protein with either the yeast GMH1, the human Gmh1, or *C. elegans* UNC-50 fused to the GAL4 activation domain (AD). A positive interaction allows yeast cells to grow on selective media lacking histidine.

GEA1/2. These genes encode large Sec7 domain-containing Arf-GEFs localized in the Golgi (Peyroche and Jackson, 2001; Garcia-Mata *et al*, 2003). A physical interaction between Gmh1p and Gea1/2p was further documented by yeast two-hybrid and immunoprecipitation experiments. We show that this interaction is conserved between UNC-50 and Gea1p, similar to the interaction between the human GMH1 and Gea1p.

How might UNC-50/GMH1 function in the trafficking of membrane proteins through the Golgi? The Gea1/2p Arf-GEFs are required for activation of the small GTPase Arf, which in turn is stably associated with Golgi and ER–Golgi intermediate compartment (ERGIC) membranes (Peyroche *et al*, 1996; Garcia-Mata *et al*, 2003). The active Arf molecule then recruits the COPI coat complex to these membranes, which leads to a further differentiation of these membranes. COPI binding to ERGIC membranes is required to polarize these membranes into domains of anterograde and retrograde cargo concentration (Shima *et al*, 1999). COPI function is also required to initiate the microtubule-dependent movement of the ERGIC toward the Golgi (Lippincott-Schwartz *et al*, 1998; Garcia-Mata *et al*, 2003). Furthermore, COPI-dependent processes are essential to maintain the integrity of the Golgi system by mediating intra-Golgi retrograde transport (Lippincott-Schwartz *et al*, 1998). It is believed that COPI-dependent processes are the main driving force for the maturation of Golgi stacks by sorting anterograde from retrograde cargo (Allan and Balch, 1999). In addition Arf activation through Arf GEFs regulates other coat protein assembly and sorting events. Clathrin-coated vesicle formation is also initiated by Arf-dependent recruitment of clathrin adaptors to Golgi subdomains (Nakayama and Wakatsuki, 2003). At this point we cannot exclude the possibility that UNC-50 also influences clathrin-dependent cargo sorting at the Golgi. However, it is unlikely that UNC-50 directly affects actin dynamics, which is also controlled by the Arf GEFs Gea1/2 in yeast (Zakrzewska *et al*, 2003), since we would expect that all receptors would be affected similarly.

The process of Arf activation, COPI coat recruitment, and subsequent GTP hydrolysis by the Arf molecules regulates the cargo concentration, sorting and uptake at specific Golgi membrane sites (Lanoix *et al*, 1999; Malsam *et al*, 1999). All these distinct coat-dependent processes are orchestrated by the activation of the small GTPases Arf through the action of Arf-GEFs. However, the Arf-GEFs do not contain any sequence for membrane attachment or TM domains. It is thus likely that integral membrane proteins like UNC-50 serve as membrane receptors to recruit Arf GEFs to distinct Golgi sites (Chantalat *et al*, 2003, 2004). Thus, recruitment of cargo proteins by an UNC-50-containing complex would provide a means to sort cargos into specific membrane domains dedicated to specific transport routes.

UNC-50 specifically affects the Lev-AChR transport

The specific sorting defect of one AChR subtype is a striking feature of the *unc-50* mutant phenotype in *C. elegans*. The *C. elegans* genome encodes up to 42 different ionotropic AChR subunits, the largest number reported in a metazoan (Jones and Sattelle, 2004). The specificity of UNC-50 for the Lev-AChR is particularly intriguing, since RIC-3, the only known factor that is required for AChR assembly within the ER, has been shown to affect the functional expression of all AChR subtypes in *C. elegans* tested so far (Halevi *et al*, 2002).

RIC-3 is necessary for cholinergic transmission mediated by the neuronal DEG-3/DES-2 AChR, the body-wall muscle levamisole- and nicotine-sensitive receptors, and the pharyngeal muscle AChR EAT-2 (Halevi *et al*, 2003). Therefore, RIC-3 most likely represents a general factor important for AChR maturation. This is in clear contrast to the AChR subtype specificity displayed by UNC-50, which does not affect the EAT-2 and DES-2/DEG-3 AChRs (data not shown) or the ACR-16-containing AChRs in muscle. Such specificity makes it highly unlikely that UNC-50 is affecting general transport in an unspecific manner that would impair AChR expression indirectly.

How is such specificity achieved? UNC-50 is conserved in organisms such as plants and yeast that do not express AChRs. In *C. elegans*, *unc-50* is ubiquitously expressed in cells outside of the nervous system that most likely do not express AChRs. This suggests that other substrates for UNC-50 may exist. However, apart from impaired movement due to the loss of the levamisole receptor, *C. elegans unc-50* mutants exhibit only marginal phenotypes including a slightly smaller body size and a low penetrance gonad migration defect (S Eimer and J-L Bessereau, unpublished observations). In line with these observations in *C. elegans*, *S. cerevisiae* deletion mutants of GMH1, the yeast ortholog of UNC-50, are completely viable and display no obvious abnormal phenotype (Chantalat *et al*, 2003). This further suggests that trafficking pathways redundant to UNC-50 might exist on a cellular level. The Lev-AChR might exclusively use this UNC-50-dependent pathway and solely rely on UNC-50-dependent transport to reach the cell surface. For example, UNC-50 might be necessary to generate a subset of Golgi membrane domains required for cell-surface trafficking of Lev-AChRs. Alternatively, UNC-50 might be required to sort a factor that directly interacts with the Lev-AChR to promote its export to the cell surface. In this respect, it is interesting to note that mutations in the *C. elegans* gene *unc-74* confer the same levamisole resistance as *unc-50* mutants and cause the same complete disappearance of levamisole-binding sites in their membranes as seen in *unc-50* (Lewis *et al*, 1987). The integration of an additional factor mediating AChR specificity would thus also partially explain why UNC-50 is evolutionary conserved, but the cargo affected is not present in every organism.

A novel mechanism for the control of ionotropic AChR expression

A tight system of control checkpoints exists to ensure that only properly folded and assembled AChRs are displayed at the cell surface. One of the best-documented control systems operates at the ER level. There, newly synthesized AChR subunits are immediately bound by the ER chaperones calnexin and Bip/Hsp70, from which they detach upon correct maturation and assembly (Forsayeth *et al*, 1992; Gelman *et al*, 1995). The association with the ER-resident chaperones retains the unassembled AChR subunits in the ER, metabolically stabilizes them, and supports their correct folding and maturation. In *C. elegans*, tandem affinity purification of tagged UNC-29 or LEV-1 subunits showed that unassembled AChRs co-purify with the ER chaperones HSP-1, HSP-3, and HSP-4, the *C. elegans* Bip/Hsp70 homologs. When the purification is designed to primarily isolate assembled AChRs, the association of LEV-1 and UNC-29 with Bip is dramatically reduced (Gottschalk *et al*, 2005). This suggests that Bip does bind unassembled Lev-AChR subunits in *C. elegans*, and that this interaction is lost during further folding and assembly of the receptor.

While the association with the ER resident chaperones may assist the correct folding of the individual subunits, a second ER-based control mechanism has been reported that ensures the pentameric assembly of the AChRs. It has been shown that unassembled AChRs are retained in the ER by a short sequence PL(Y/F)(F/Y)XXN present in the first TM domain of the α4 subunit that is buried upon full assembly of the pentamer (Wang *et al*, 2002). This sequence can also be found in all of the Lev-AChR subunits with minor alterations (S Eimer, unpublished observation). Therefore, it is likely that in *C. elegans*, unassembled Lev-AChR subunits and receptors are also retained within the ER. These unassembled receptors can be stably detected in membrane extracts of mutants in which one of the Lev-AChR subunits is not produced. This is in agreement with previous reports showing that endogenous ER-retained receptors are metabolically stable in mouse myotubes (Gu *et al*, 1989), as well as when one subunit is missing (Black *et al*, 1987). Additionally, an Arg–Lys motif in the large cytoplasmic loop of the subunits has been implicated in Golgi-to-ER retrieval of subunits and unassembled complexes that have escaped out of the ER (Keller *et al*, 2001; Ren *et al*, 2005). This work suggests that an additional control mechanism may exist that operates within the ER–Golgi intermediate compartment to prevent immature AChRs from reaching the cell surface.

Our analysis of *C. elegans unc-50* mutants revealed a regulation of AChR biogenesis that operates after AChR assembly. The assembled Lev-AChR has the option either to be delivered to the cell surface or to enter the lysosomal route and be degraded. We showed here that UNC-50 function is required to sort the Lev-AChR to the cell surface and, at the same time, prevent the receptor from entering the lysosomal route and degradation. Since the yeast ortholog of UNC-50, Gmh1p, was demonstrated to cycle from the Golgi to the ER, we cannot exclude the possibility that in the absence of UNC-50, assembled Lev-AChRs bypass the Golgi and are directly sent to lysosomes. Alternatively, UNC-50 might achieve its function within the Golgi apparatus by supporting a COPI-dependent sorting event, which would be necessary to keep the Lev-AChR *en route* to the plasma membrane. Several mechanisms can be used to sort defective proteins for lysosomal degradation after they exit the ER (reviewed in Arvan *et al*, 2002). It was recently shown in tobacco that the chaperone BiP can exit the ER with a subpopulation of ligands and target them to the lytic vacuole (Pimpl *et al*, 2006). One possible scenario could be that UNC-50, by recruiting Arf-GEFs to a subset of Golgi membranes, would sort a factor away from the receptor into the COPI-dependent retrograde route that otherwise would tag the Lev-AChR for lysosomal degradation.

The analysis of AChR synthesis in *unc-50* mutants has unmasked a previously unsuspected degradative pathway for nicotinic receptors. The existence of post-assembly sorting from a degradative pathway would be worth exploring for other ligand-gated ionotropic receptors, since shifting the destiny of assembled receptors toward either degradation or cell-surface delivery would provide a means to rapidly modify the number of receptors delivered at the cell surface under

physiological or pathological conditions. Genetic strategies in *C. elegans* will provide a means to further dissect the mechanisms of UNC-50-dependent AChR trafficking.

Materials and methods

Strains and genetics

C. elegans strains were cultured as described previously (Brenner, 1974) and kept at 20°C, if not otherwise stated. The following mutations and combinations thereof were used in this study: LG I: *unc-29(x29), unc-38(x20), unc-63(kr13, x37);* LG III: *cup-5(ar465)*, *unc-50(e306, x35, x47, x515)*; LG IV: *lev-1(kr6)*; LG X: *lin-15 (n765ts)*. All mutations are described in wormbase (www.wormbase.org), unless otherwise noted.

Expression constructs and transgenic animals are described in Supplementary data.

Electrophysiology

Electrophysiological methods were performed as described previously (Richmond and Jorgensen, 1999). Further details are provided in Supplementary data.

Antibodies and immunostaining

Antibodies against UNC-29 were generated and purified as described previously (Gally *et al*, 2004). For immunostaining experiments, mixed-stage worms were fixed with methanol/acetone and stained according to the freeze fracture protocol (Gally *et al*, 2004).

Protein extraction and Western blotting

Protein extracts were prepared and probed as described (Gally *et al*, 2004; see Supplementary data).

Test for levamisole sensitivity and movement

To test the sensitivity to levamisole, well-fed young adult hermaphrodites were transferred onto plates containing 1 mM levamisole and assayed for lack of hypercontraction and their ability to move after 1 h. To quantitatively assess the movement of wild-type and mutant animals, worms were transferred to unseeded plates and after a two 2-min interval of adjustment the body bends per minute (corresponds to a whole 360° sine wave) were counted over a 1-min time interval. For each animal tested, five consecutive intervals were averaged.

Two-hybrid analysis

The interaction test of yeast proteins GMH1 and GEA1 within the two-hybrid system have been described previously (Chantalat *et al*, 2003). The GEA1(aa 749–1408) GAL4 DNA-binding domain (DB) fusion construct was kindly provided by A Peyroche and S Chantalat. pGADT7 (Clontech) was used to construct the fusions of UNC-50, GMH1, and hGmh1 to the GAL4 activation domain. The constructs were transformed into strain AH109 (Clontech) and analyzed according the manufacturer's protocol. As a negative control, the L862S mutation was introduced into GEA1(aa 749–1408) GAL4DB construct and the interactions with UNC-50, GMH1, and hGmh1 were assayed again. The L862S mutation has been shown to strongly reduce the interaction between Gmh1 and Gea1 (Chantalat *et al*, 2003).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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